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Optimization of ultrasound-assisted extraction of natural antioxidants from *Piper betle* using response surface methodology

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Abstract

Natural antioxidants are excellent substitute for their synthetic counterparts in dietary supplements. This study employed three-level Box-Behnken design through RSM to optimize the recovery of natural antioxidants from *Piper Betle via* ultrasound-assisted extraction (UAE). The influence of three extraction parameters, temperature (50-70 ^{o}C), ethanol concentration (70-90%) and solute to solvent ratio (1:10 – 1:30 g/mL) on the extraction yield (EY), total phenolic content (TPC) and antioxidant capacity was investigated. The optimum conditions were determined to be 51.60 ^{o}C with 78.74% ethanol and ratio of 1:21.85 g/mL. Experimental validation showed maximum EY of 13.88% with TPC of 311.21 mgGAE/gDW and 97.57% antioxidant capacity that were all within 95% confidence level of predicted values. Additionally, UAE gave significantly better yield (13.71%), TPC (289.05 mgGAE/gDW), total flavonoid content (21.50 mgRE/gDW) and antioxidant activity (94.99%) than maceration which gave yield (10.96%), TPC (246.98 mgGAE/gDW), total flavonoid content (13.48 mgRE/gDW) and antioxidant activity (78.12%). General phytochemical screening exposed the presence of additional saponins and tannins in the UAE extracts. Chemical composition of the optimized extract *via* GC/MS indicated the presence of four major phenolic compounds, hydroxychavicol, eugenol, isoeugenol and 4-allyl-1,2-diacetoxybenzene with peak areas of 66.55, 11.92, 2.90 and 3.21% respectively.

List of compounds

Hydroxychavicol (PubChem CID: 70775)

Eugenol (PubChem CID: 3314)

Isoeugenol (PubChem CID: 853433)

4-Allyl-1,2-diacetoxybenzene (PubChem CID: 166872)

1. Introduction

Piper betle, belonging to the *Piperaceae* family, are the leaves of a woody plant that is widely distributed mainly across Asian regions. In traditional Asian medicine, *Piper betle* is known as one of the most common medicinal plants utilized as contemporary and alternative medicine among cancer patients (Farooqui et al., 2016). The herb's effective antioxidant potential has been demonstrated *via* multiple radical scavenging activities (Sazwi, Nalina, & Rahim, 2013). Furthermore, the extract of *Piper betle* has been proven to reduce and inhibit lipid peroxidation together with enhancing the levels of natural antioxidants such as Vitamin C and E (Saravanan, Prakasam, Ramesh, & Pugalendi, 2004). The reason behind the antioxidative nature of *Piper betle's* extract is due to the existence of natural antioxidants like hydroxychavicol and eugenol (Chakraborty & Shah, 2011; Pin et al., 2010). Due to its efficacy, researchers have proposed the possible utilization of *Piper betle* as a source of natural antioxidants in food and pharmaceutical products (Dwivedi & Tripathi, 2014; Venkadeswara et al., 2014).

Conventional extraction methods such as distillation and solvent extraction (maceration, soxhlet, percolation, infusion extraction) and non-conventional ones including supercritical fluid extraction, accelerated solvent are typically implemented in the recovery of natural antioxidants (Azwanida, 2015). As effective as they may be, high solvent and energy consumption and prolonged extraction period makes them undesirable from the economics' perspective (González-Centeno, Comas-Serra, Femenia, Rosselló, & Simal, 2015). The use of ultrasound in the recovery of desired compounds has been proven to be an effective and efficient extraction technique in terms of garnering more yield with reduced solvent usage and extraction time (Vilkhu, Mawson, Simons, & Bates, 2008). Ultrasound-assisted extraction (UAE) relies on the phenomenon of acoustic cavitation and mechanical effects for the extraction of compounds from plants sources. Collapse of the cavitational bubbles on the plant matrix's surface causes the cell walls to rupture, resulting in higher and faster penetration of the solvent into the plant material. Thus, due to enhanced overall mass transfer, the extraction of the desired compounds are accelerated (Tomšik et al., 2016; Vilkhu et al., 2008).

The use of ultrasound for extraction applications in food and pharmaceutical industries is promising. However, the utilization of ultrasound for the recovery of natural antioxidants from the medicinal herb, *Piper betle*, is yet to be fully explored. Thus, the primary aim of this paper is to optimize the ultrasound-assisted extraction of natural antioxidants from *Piper betle*. This was achieved by investigating the impact of three extraction parameters (temperature, solute to solvent ratio and solvent concentration) for optimum extraction yield, TPC and 2,2-diphenyl-1-picrylhydrazy (DPPH) antioxidant capacity. The statistical approach of response surface methodology was employed for the optimization of extraction parameters. This paper also aims to draw comparison between the phenolic content and antioxidants activities of *Piper betle* using UAE and conventional maceration method. Consequently, this paper also aims to identify and quantify the predominant phenolic compounds present in the optimized *Piper betle's* extract that contributes to the high antioxidant activity of *Piper betle via* Gas chromatography–mass spectrometry (GC/MS).

2. Materials and methods

2.1. Plant materials

A total of 10 kg of fresh leaves of *Piper betle* were purchased in a single batch from a local shop in Chow Kit market, Kuala Lumpur, Malaysia. The washed and cleaned leaves were pre-treated (dried) in an air forced convection oven (FAC-350, Protech, USA) at 50 ^{o}C for a day. The dried leaf samples were then crushed into powdered form and conceded through 800 µm-mesh sieve before being used for actual extraction.

2.2. Chemicals and reagents

The two reagents Fast blue BB (FBBB) and DPPH (1,1-diphenyl-2-picrylhydrazyl) of analytical grade were purchased from Sigma-aldrich (Sigma-aldrich GmbH, Steinheim, Germany). The solvents used in this research include 95% ethanol, 99.9% methanol and chloroform (HPLC grade). The remaining chemicals used include Gallic acid standard and sodium hydroxide pellets. All of the chemicals mentioned with the exception of the reagents were purchased from Sigma-aldrich (Sigma, St. Louis, MO, USA).

2.3. Extraction procedures

2.3.1 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction of phenolic compounds from *Piper betle* was performed using an ultrasonic bath system (P120 H, Elmasonic, Germany). 1 g of powdered leaf with the designed volume of ethanol concentration were placed in an ultrasonic bath that is equipped with digital control system for sonication time, temperature and frequency. Based on the experimental design, UAE was performed at a frequency of 37 kHz with a constant power of 400 W. Extraction temperature and time were continuously monitored from the control panel of the equipment. Distilled water was added to maintain a constant desired temperature with ±2 ^{o}C in the ultrasonic bath. Extraction period of 30 minutes was applied based on preliminary trial studies as prolong extraction time can lead to structural alteration and disintegration of the bioactive compounds (Moorthy et al., 2017). The impact of extraction temperature (50, 60 and 70 ^{o}C), solvent concentration (70%, 80% and 90% v/v) and solute to solvent ratio (1:10, 1:20 and 1:30 g/mL) were investigated. Following the extraction, samples were filtered and dried at 50 ^{o}C using a vacuum rotary evaporator (Hei-VAP Platinum 3, Heidolph, Germany) to obtain the crude extract. The crude extracts were stored at 4 ^{o}C prior to consequent analysis.

2.3.2 Maceration extraction

Maceration extraction of the phenolic antioxidants from *Piper betle* was performed in a water bath system (Copens Scientific Sdn Bhd, Malaysia). 1 g of powdered and sieved leaf samples were extracted with 80% ethanol at 50 ^{o}C for 30 minutes. The extracts obtained were dried in the same manner as above and stored at 4 ^{o}C before further analysis.

2.4. Total phenolic content (TPC) and extraction yield

Extraction yield (EY) of the crude extract was obtained using Eq. (1). TPC was quantified as described by Medina (2011) with slight modifications. 1:20 mg/mL of crude extract in deionized water was added to 0.1 mL of 0.1% FBBB reagent which was kept aside for a min. This was followed by the addition of 0.1 mL of 5% sodium hydroxide solution. The mixtures were kept at room temperature for 90 minutes before transferring 200 µL of the sample mixtures to a 96-well plate. The absorbance of the samples were read at 420 nm by means of a microplate spectrophotometer (Epoch 2, BioTek, USA) (Medina, 2011). TPC is expressed in terms of mg gallic acid equivalent/g of dried extract according to the regression equation of gallic acid calibration curve (r^2 = 0.9899) that was procured in the same manner as above.

$$EY = \frac{W_d}{W_s} \times 100\% \quad (1)$$

Where W_d and W_s are the weight of the crude extract and *Piper betle* powder sample in grams respectively.

2.5. Total flavonoid content (TFC) and phytochemical screening

Total flavonoid content (TFC) assay was conducted according to Ayoola *et al.* (2008) with minor modifications. 2 ml of extract samples with concentration of 1 mg/mL was added to 2 mL of 2% aluminium trichloride ethanolic solution. The sample mixtures were kept at room temperature for an hour before measuring their absorbance at 420 nm *via* a microplate spectrophotometer (Ayoola et al., 2008). TFC is expressed in terms of mg rutin equivalent/g of dried extract according to the regression equation of rutin calibration curve (r^2 = 0.9839). The general phytochemical screening of alkaloids, steroids, polysaccharide, condensed tannins and saponins were performed as elaborated by Adline and Devi (2014) and Evans (2009).

2.6. DPPH antioxidant assay

A modified version of DPPH radical scavenging assay was followed as described by Pin *et al.* (2010). Samples mixtures were prepared in concentrations of 0.5 mg/mL in 80% ethanol. Aliquots of 160 µL of *Piper betle* samples mixture were transferred to 96-well plate which was followed by the addition of 40 µL of working 1mM DPPH methanolic solution. The plates were kept in the dark for 3 min in ambient temperature. The absorbance of the sample solutions were read at 520 nm with a microplate spectrophotometer. The radical scavenging activity, is expressed as % inhibition activity with the following Eq. (2) (Pin et al., 2010):

$$\text{DPPH \% Inhibition activity} = \frac{A_c - A_S}{A_c} \times 100 \quad (2)$$

Where A_c is the absorbance of blank solution containing DPPH only and A_s is the absorbance of the solution containing DPPH with *Piper betle* extract.

2.7. Gas chromatography/Mass spectroscopy (GC/MS) assay

Chemical composition of the optimized extract samples were performed as elaborated by Foo, Salleh, & Mamat (2015) using GC/MS (7890A, Agilent Technologies, Malaysia) with slight modifications. Initial temperature of the oven was programmed at 70 ^{o}C that was raised to 305 ^{o}C at a rate of 20 ^{o}C/min. Helium (carrier gas) was injected at a rate of 1.2 mL/min. 1 mL of 0.1 mg/mL samples were injected into the capillary column in split mode for run time of approximately 17 min (Foo, Salleh, & Mamat, 2015). Identification of the individual compounds was done by library match with NIST Mass Spectral library (version 2).

2.8. Response surface methodology (RSM)

In present study, a three-factor, three-level Box-Behnken design (BBD) was employed to obtain the optimum UAE conditions for the extraction of antioxidants from *Piper betle*. BBD was selected for current research as it is particularly effective when three variables are concerned in the experimental domain with reduced number of experiments allowing for a more efficient and economic approach (Granato & Ares, 2013). The extraction variables with their respective levels and coded factors are displayed in Table 1. The complete design matrix of BBD with a total of 17 experiments is presented in Table 2. Experimental data of predicted and actual responses were collected in the form of extraction yield, TPC and DPPH antioxidant activity (Table 2). The experimental data for the three responses were fitted into second-order polynomial model as in the following equation:

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_j \sum_{i=2}^{k} \beta_{ij} x_i x_j \quad (3)$$

Where Y is the response, x_i and x_j are the independent variables (i and j range from 1 to k), β_o is a constant, $\beta_{i,}$ β_{ii}, and β_{ij} are the regression coefficients of linear, quadratic and interactive terms respectively, k is the number of number of parameters (3 for current study) (Moorthy et al., 2017).

2.9. Statistical analysis

All of the analysis above were carried out in triplicates and values expressed as mean. Regression analysis of the experimental data was performed using Design expert software v. 10 (Stat-Ease, Minneapolis, Minnesota, USA). Analysis of variance (ANOVA), different statistical parameters including coefficient of determination (R^2), adjusted coefficient of determination (R^2_a) and predicted coefficient of determination (R^2_p) were all employed to check the adequacy of the developed models. Coefficient of

variation (*CV*) and adequate precision were also examined to further evaluate the precision of the developed models. Significance of each term was considered when $\rho<0.05$. In addition to the quadratic models, response surface plots were generated to establish the relationship between the independent variables and the responses.

2.10. Optimization and validation of RSM extraction models

Numerical optimization technique was performed to determine the optimum conditions for maximum EY, TPC and DPPH antioxidant activity. The prime conditions were identified with the desirability value of 1 for each respective response. Validation of the developed models were done by performing triplicate experiments under the optimized parameters. Finally, the average experimental results and 95% prediction interval range of predicted values were compared. This is essential to evaluate the accuracy and precision of the optimized conditions.

3. Results and discussion

3.1. Determination of extraction parameters for RSM optimization

When solvent extractions are concerned, the selection of appropriate solvent is as pivotal as any other extraction parameter. Organic solvents are among the best when it comes to the recovery of phenolic compounds from *Piper betle*, namely ethanol and methanol (Nouri, Nafchi, & Karim, 2014). Preliminary studies done with a variety of solvents validates the effectiveness of ethanol in extracting the phenolics from *Piper Betle*. Moreover, ethanol is commended for practical usage as it is a safe, low toxic and eco-friendly solvent that is reusable and generates less wastes (Dias et al., 2017; Zhao et al., 2014). Hence, ethanol was deemed a better option since this research involves the potential use of phenolic compounds in pharmaceutical and food industrial applications.

Three independent variables and their respective levels were selected for RSM optimization in current study: temperature (50, 60 and 70 ^{o}C), ethanol concentration (70, 80, 90%) and solute to solvent ratio (1:10, 1:20, 1:30 g/mL). The parameters and their respective ranges investigated were based on conventional extraction technique of maceration and soxhlet (Keshani, Abdullah, Mobarekeh, Rahman, & Bakar, 2010; Muruganandam, Krishna, Reddy, & Nirmala, 2017; Nouri and Mohammadi Nafchi, 2014; Pin et al., 2009).

3.2. Comparison of ultrasound-assisted extraction with conventional extraction method

Maceration is a common extraction technique that has been employed numerous times by other researchers for the extraction of bioactive compounds from *Piper betle* (Nouri & Mohammadi Nafchi, 2014; Pin et al., 2010; Rathee, Patro, Mula, Gamre, & Chattopadhyay, 2006). However, newer techniques such as ultrasound extraction are yet to be implemented for this particularly potent medicinal herb. This paper aims to draw comparison between the two extraction technique's effectiveness for the recovery

of antioxidant agents from *Piper betle*. Multiple analysis were executed including EY, TPC, TFC and DPPH antioxidant activity with additional phytochemical screening of alkaloids, steroids, polysaccharides, tannins and saponins (Table 3 and 4). The results disclosed a maximum EY of 13.71% was recovered from *Piper betle* with the aid of ultrasound, while, maceration resulted a lower yield of approximately 10.96%. Likewise, the results also revealed UAE to have significantly higher TPC (289.05 mgGAE/gDW), TFC (21.5 mgRE/gDW) and superior antioxidant activity with 94.99% inhibition in comparison to maceration that gave noticeably lower TPC (246.98 mgGAE/gDW), TFC (13.58 mgRE/gDW) and 78.12% antioxidant activity. The noteworthy improvement could be attributed to the acoustic cavitation of ultrasound and its mechanical effects that resulted in better recovery. The outward shockwave produced from the implosion of the cavitation bubbles generates macro-turbulence and high-velocity inter particle collision. This in turn facilitates diffusion and overall mass transfer of the system. Cavitation occurring near the surface of the plant's cell results in surface peeling, cell breakdown and erosion that further accentuates the recovery process (Pico, 2013; Vilkhu et al., 2008). The accumulating effect of multiple mechanisms arising from acoustic cavitation ultimately leads to enhanced recovery of the desired compounds.

The effectiveness of ultrasound was also noticeable in the general phytochemical screening where additional phytoconstituents of tannins and saponins were detected in the UAE extracts only. On the otherhand, similar amounts of steroids were detected in both UAE and maceration extracts. Bioactive compounds such as tannins, steroids and flavonoids have all been identified as major sources of antioxidants (Vaithiyanathan & Mirunalini, 2015). Particularly, flavonoids and its derivatives have been established as excellent free radical scavengers. Research has shown saponins and tannins to be potent anti-inflammatory agent with the latter known to be highly effective in the prevention of cancer (Wintola & Afolayan, 2011). Saponin was also found to be part of plant's defence mechanism due to its anti-microbial properties (Alabri, Musalami, Hossain, Weli, & Al-Riyami, 2014). All of the additional bioactive compounds detected in the UAE extracts have contributed to its remarkable antioxidant activities. Therefore, it can be said with certainty that UAE is comparatively a superior extraction method for the recovery of natural antioxidants from *Piper betle.*

3.3. Influence of extraction parameters on extraction yield

It is crucial to analyze the influence of extraction parameters in order to effectively isolate and utilize the compounds of interest. Therefore, a three-level, three-factor BBD was employed to investigate the effect of various independent extraction variables on the optimal recovery of phenolic compounds from *Piper betle*. Regression analysis of all three responses are presented in Table 5. The evaluation of the linear terms revealed solute to solvent ratio to have significant positive influence on EY. On the contrary, both extraction temperature and ethanol concentration had significant negative effects on the response. Interaction between solute to solvent ratio and ethanol concentration displayed a slight positive effect, however, the quadratic effect of solute to solvent ratio

was significantly negative. The remainder of the terms were not significant, therefore, were excluded from the final model in Eq. (4).

$$Y_1 = 11.91 - 1.74X_1 + 0.94X_2 - 0.41X_3 + 0.60X_{23} - 1.41X_2^2 \quad (4)$$

Figure 1 shows the response plots of extraction yield generated by varying two variables at a time. This is crucial to illustrate the effects of the independent variables on extraction yield. The plots are in good agreement with regression analysis as the positive linear influence of solute to solvent ratio is clearly noticed with maximum EY recovered at 1:20 g/mL. The presence of more ethanol in the extraction solution creates a larger concentration gradient. This acts as a driving force for higher diffusion of solvent into the plant cells, thereby, improving the overall mass transfer of the system (Charpe & Rathod, 2014). Moreover, increased amount of ethanol enhances the contact area between the solvent and the solute, thus, improving the solubility of the phenolic compounds from within the plant cells (Moorthy et al., 2017; Xu et al., 2017). Taking the quadratic terms into account, the negative influence of solute to solvent ratio can also be accounted for in the response plots where a saddle curve is observed. UAE is highly dependent on the effects of acoustic cavitation for the formation and rupture of bubbles to facilitate the mass transfer of the process. Further increase in the ratio may hamper with the dispersion of the ultrasound energy density throughout the solution, hence, negatively effecting EY (Moorthy et al., 2017). Based on ANOVA (Table 5), the developed model was found to be significant at an *F-value* of 62.79. High value of correlation coefficient (R^2 = 0.9878) confirms the validity of the deduced model and its ability to describe the relation between the variable and the response. The value of adjusted correlation coefficient (R^2_a = 0.9721) being very close to R^2 confirms high significance of the deduced model. High predicted correlation coefficient (R^2_p = 0.8068) further implies the model's adequacy to predict the relation (Maran, Sivakumar, Sridhar, & Immanuel, 2013). Coefficient of variation of 2.5% (*CV*<10%), not only indicates low deviation between the experimental and predicted values, but also a high degree of precision and reliability (He et al., 2016). Adequate precision of 28.36 indicates good signal and competent model fitness (Maran, Manikandan, Thirugnanasambandham, Nivethaa, & Dinesh, 2013).

3.4. Influence of extraction parameters on total phenolic content (TPC)

Judging of the regression analysis of the linear terms from Table 5 shows the impact of both temperature and solute to solvent ratio on TPC were of high significance. Furthermore, all three extraction parameters have shown a concrete negative influence on the quadratic terms. For the response of TPC, interaction between temperature and solute to solvent ratio showed moderately significant negative effect. All of the other terms including remaining two interactions were insignificant. Thus, they were excluded from the final developed model as expressed in Eq. (5).

$$Y_2 = 301.66 - 38.75X_1 + 14.47X_2 - 16.47X_{12} - 28.70X_1^2 - 62.81X_2^2 - 60.03X_3^2 \quad (5)$$

All of the experimental results and response plots presented in Table 5 and Figure 2 indicate ratio and temperature had a positive and negative influence respectively. The initial increase in TPC may be a result of enhanced solubility due to decreased intermolecular interactions within the solvent caused by high temperatures (Jianming, Yuan, Ping, Feng, & Liying, 2013). Moreover, reduced solvent viscosity caused by the thermal effect lead to improved solubility of the solvent into the plant matrix (Moorthy et al., 2017; Xu et al., 2017). At the same time, thermal degradation of the phenolic compounds was the most likely reason behind the decrease of TPC at high temperatures beyond 52 ^{o}C (Dranca & Oroian, 2016; Tomšik et al., 2016). The thermo-sensitive nature of the phenolics in *Piper betle* has been previously noted. Eugenol, a common phenolic in *Piper betle's* extract, was found to decrease when applied extraction temperature was higher than 60 ^{o}C (Pin et al., 2009). Results disclosed by the authors are in agreement with current study that saw a similar decrease in TPC with increasing temperature.

Based on the statistical analysis, the developed model was found to be significant at an *F-value* of 61.85. High values of R^2 (0.9795) and R^2_a (0.9636) indicates high degree of correlation. The predicted correlation coefficient (R^2_p = 0.8401) was also determined to be of high significance. In addition, values of coefficient of variation (*CV* = 4.85) and adequate precision (adeq. precision = 18.262) further indicates the ability of the deduced model to define the relation between the extraction variables and the response of TPC.

3.5. Influence of extraction parameters on DPPH antioxidant capacity

The results obtained indicate all three extraction parameters to have significant linear as well as quadratic effect on the antioxidant activity. Further evaluation also exposes no significant effect by any interaction terms on the response. The final developed model excluding the non-significant terms are given in Eq. (6).

$$Y_3 = 94.71 - 11.88X_1 + 4.23X_2 - 2.89X_3 - 7.92X_1^2 - 13.70X_2^2 - 14.75X_3^2 \quad (6)$$

The negative effect of extraction temperature is clearly visible in Figure 3 as increasing temperature results in lower antioxidant activity. Like the previous two responses of EY and TPC, solute to solvent ratio seems to have a moderate positive effect on the antioxidant activity. On the contrary, ethanol concentration was found to have a more profound impact for this response only. This suggests its major role in the extraction of antioxidant agents from *Piper betle*. The solubility and extractability of polar phenolic compounds are better with polar solvents (Tomšik et al., 2016). However, the impact of solvents on the recovery of antioxidants is very much dependent on the composition of the solvents, provided it is a dual solvent mixture. According to Mustafa and Turner (2011), the rule of thumb for the choice of solvents is the principle of "*like dissolve like*". Solvents tend to solubilize compounds with similar properties much more easily (Mustafa & Turner, 2011). Thereby, it can be assumed that the polarity of the phenolic antioxidants in *Piper betle* are closer to that of ethanol. As a result, the extraction of antioxidants increased with higher ethanol concentrations with the maximum recovery obtained at 80% ethanol concentration.

Considering the statistical analysis, the developed model was found to be valid for an *F-value* of 70.18. High correlation coefficients of R^2 (0.9890), R^2_a (0.9749) and R^2_p (0.8246) indicates the model's ability to represent the extraction process. Low value of *CV* (3.05) and high value of adeq. precision (21.516) further confirms the model's ability for expressing the antioxidant capacity of *Piper betle's* extract.

3.6. RSM optimization and model validation

Several numerical optimizations were performed to identify the best possible combination that can achieve the desired output. The optimized condition was determined at 78.74% ethanol concentration with ratio of 1:21.85 g/mL at a temperature of 51.60 ^{o}C. The experimental results produced an extraction yield of 13.88% with a TPC of 311.21 (mgGAE/gDW) and 97.57% antioxidant activity. The results were well in the range of 95% prediction intervals that were obtained from the developed second-order models (Table 6). Good correlation between the predicted and experimental responses confirm the models obtained can accurately predict the ultrasound-assisted extraction of phenolic antioxidants from *Piper betle*.

3.7. Chemical composition and quantitative analysis of the optimized extract

GC/MS analysis was performed to determine the chemical composition and quantity of the phenolic antioxidants in the optimized extract (Table 7 and Figure 4). The analysis revealed the presence of hydroxychavicol (peak 2) which was found to be the dominant component with 66.55% peak area. It was followed by eugenol (peak 1 with 11.92%), 2-methoxy-4-propenyl-acetate (peak 3 with 2.90%) and 4-allyl-1,2-diacetoxybenzene (peak 4 with 3.21%) with concentrations of 0.067, 0.012, 0.003 and 0.003 mg/mL respectively. Hydroxychavicol has been reported to be the major phenolic compound present in ethanolic extract of *Piper betle via* HPLC (Pin et al., 2010). Its antioxidant status has also been explored by other researchers (Chang et al., 2002; Sharma et al., 2009). At the same time, both eugenol and isoeugenol were also found to prevent DNA oxidation and lipid peroxidation, damaging reactions caused by free radicals that leads to oxidative stress (Atsumi, Fujisawa, & Tonosaki, 2005; Nam & Kim, 2013). 4-allyl-1,2-diacetoxybenzene, commonly referred as allylpyrocatechol 3,4-diacetate, is another major phenolic compound present in *Piper betle* (Arambewela, Arawwawala, & Ratnasooriya, 2005; Muruganandam et al., 2017). Although very little literature exists on the antioxidant potential of this compound, a study revealed allylpyrocatechol 3,4-diacetate to possess protective and scavenging properties against free radicals and lipid peroxidation (Bhattacharya et al., 2007). The presence of three major phenolics with high antioxidant potential revealed through GC/MS enhances the possibility of using *Piper betle's* extract as natural antioxidant agents in the food industry.

4. Conclusion

Present study successfully employed UAE to extract natural antioxidants from *Piper betle* by investigating the influence of three extraction parameters: temperature, solvent concentration and solute to solvent ratio *via* BBD. In general, all parameters were found to have significant impact on the responses with ethanol concentration specifically affecting the antioxidant activity. The optimized condition was determined at 78.74% ethanol concentration with solute to solvent ratio of 1:21.85 g/mL at 51.60 ^{o}C. Under the optimum conditions, maximum yield of 13.88% was retrieved with TPC and antioxidant activity of 311.21 mgGAE/gDW and 97.57% inhibition respectively. Using the mathematical approach of RSM, second-order polynomials models were developed for the responses of extraction yield, TPC and DPPH antioxidant capacity. Statistical analysis of high correlation coefficients confirms the validity of the proposed models. Validation of the optimized conditions also reveals little deviation as the experimental values obtained were well within 95% prediction interval.

Additionally, comparative research confirmed the extraction of secondary metabolites including tannins, saponins and flavonoids together with phenolic antioxidants using UAE was significantly higher than maceration. High phenolic content that corresponds with equally effective antioxidant potential solidifies UAE as an efficient and practical extraction method for the recovery of natural antioxidants from *Piper betle*. Further analysis of the optimized UAE extract through GC/MS reveals the presence of four major phenolic compounds: hydroxychavicol, eugenol, isoeugenol and 4-allyl-1,2-diacetoxybenzene with peak area of 66.55%, 11.92%, 2.90% and 3.21% respectively.

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Figure captions

Fig. 1. 3D Response surface plots demonstrating the effects of different extraction parameters on extraction yield (a) ethanol concentration and temperature (b) ethanol concentration and solute to solvent ratio (c) solute to solvent ratio and temperature

Fig. 2. 3D Response surface plots demonstrating the effects of different extraction parameters on TPC (a) ethanol concentration and temperature (b) ethanol concentration and solute to solvent ratio (c) solute to solvent ratio and temperature

Fig. 3. 3D Response surface plots demonstrating the effects of different extraction parameters on DPPH % inhibition capacity (a) temperature and ethanol concentration (b) ethanol concentration and solute to solvent ratio (c) solute to solvent ratio and temperature

Fig. 4. GCMS chromatogram of the optimized *Piper Betle's* extract (peak 1: eugenol; peak 2: hydroxychavicol; peak 3: isoeugenol; peak 4: allylpyrocatechol 3,4-diacetate)

Table 1. Experimental domain for Box-Behnken design

Variable	Factor levels		
	-1	0	1
Temperature (X_1, °C)	50	60	70
Ratio (X_2, g/mL)	1:10	1:20	1:30
Concentration (X_3, %)	70	80	90

Table 2. Box-Behnken design matrix with experimental and predicted responses[a]

Run	Temperature (^{o}C)	Ratio (g/mL)	Concentration (%)	EY (%)		TPC (mgGAE/gDW)		DPPH (% inhibition activity)	
				Actual response	Predicted response	Actual response	Predicted response	Actual response	Predicted response
1	60	30	90	11.43±0.42	11.50	173.85±0.35	190.76	64.41±0.06	67.43
2	50	10	80	11.42±0.25	11.23	206.41±0.21	217.96	78.51±0.07	79.38
3	70	30	80	9.43±0.26	9.61	180.95±0.57	169.40	64.94±0.10	64.07
4	50	30	80	12.73±0.31	12.83	286.61±0.65	279.83	92.03±0.04	90.57
5	60	20	80	11.92±0.53	11.91	300.75±0.57	301.66	94.65±0.02	94.71
6	60	10	90	8.08±0.13	8.43	168.41±0.49	161.81	58.60±0.18	59.30
7	60	20	80	11.89±0.16	11.91	302.28±0.10	301.66	94.78±0.02	94.71
8	60	20	80	11.98±0.25	11.91	299.65±0.58	301.66	94.69±0.08	94.71
9	70	20	70	10.33±0.15	10.50	175.45±0.55	176.72	63.11±0.03	64.68
10	60	20	80	11.91±0.36	11.91	302.88±0.10	301.66	94.72±0.08	94.71

11	60	30	70	11.47±0.23	11.12	194.41±0.67	195.83	74.24±0.04	73.54
12	60	20	80	11.83±0.22	11.91	302.75±0.55	301.66	94.70±0.03	94.71
13	70	20	90	9.43±0.49	9.18	168.15±0.06	171.64	57.79±0.12	55.63
14	50	20	70	13.24±0.15	13.49	245.18±0.36	254.22	83.02±0.18	85.18
15	60	10	70	10.51±0.46	10.45	178.61±0.72	166.89	67.77±0.04	64.75
16	50	20	90	13.33±0.32	13.16	262.95±0.35	249.14	84.23±0.06	82.66
17	70	10	80	7.57±0.52	7.47	166.61±0.55	173.39	56.88±0.13	58.34

[a]Values are expressed as mean ± standard deviation (n = 3)

Table 3. Extraction yield, total phenolic content and total flavonoid content of *Piper betle* extracts with UAE and maceration[a]

Response	Extraction yield	TPC (mgGAE/gDW)	TFC (mgRE/gDW)	DPPH (% inhibition activity)
UAE	13.71±0.23	289.05±0.57	21.5±0.21	94.99±0.15
Maceration	10.96±0.14	246.98±0.34	13.48±0.26	78.12±0.18

[a]Values are expressed as mean ± standard deviation (n = 3)

Table 4. General phytochemical screening of *Piper betle* extracts with UAE and maceration[a]

Phytoconstituents	UAE	Maceration
Alkaloids	-	-
Saponins	++	-
Tannins	++	+
Steroids	++	++
Polysaccharides	-	-

[a] (+) Present (++) Present in high amount (-) Absent

Table 5. Estimated regression coefficients and Analysis of variance (ANOVA) for the investigated parameters

Term	Estimated regression coefficients					
	EY	ρ-value	TPC	ρ-value	DPPH	ρ-value
Intercept β_o	11.91		301.66		94.71	
X_1	-1.74	<0.0001	-38.75	<0.0001	-11.88	<0.0001
X_2	0.94	<0.0001	14.47	0.0052	4.23	0.0015
X_3	-0.41	0.004	-2.54	0.5363	-2.89	0.0107
X_{12}	0.14	0.3616	-16.47	0.0162	-1.37	0.2865
X_{13}	-0.25	0.1167	---		-1.63	0.2103
X_{23}	0.60	0.0034	---		-0.16	0.8940
X_1^2	-0.20	0.1747	-28.70	0.0005	-7.92	0.0002
X_2^2	-1.41	<0.0001	-62.81	<0.0001	-13.70	<0.0001
X_3^2	-0.12	0.4034	-60.03	<0.0001	-14.75	<0.0001
Model F-value	62.97	<0.0001	61.85	<0.0001	70.18	<0.0001
Mean	11.09		230.35		77.59	
C.V. %	2.50%		4.85%		3.05%	
Adeq. precision	28.355		18.262		21.516	
R^2	0.9878		0.9795		0.9890	
R^2_a	0.9721		0.9636		0.9749	
R^2_p	0.8068		0.8401		0.8246	

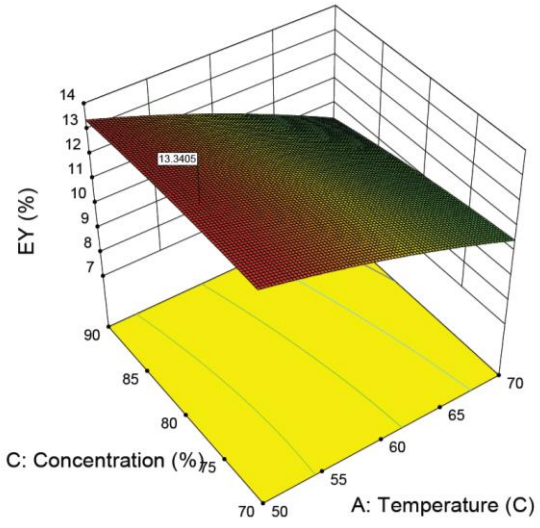
Table 6. Predicted and obtained response values and confidences[a]

Response	Predicted response	95% PI low	Obtained response	95% PI high
EY (%)	13.340	12.603	13.880±0.34	14.078
TPC (mgGAE/gDW)	316.411	287.986	311.210±0.25	344.835
DPPH (% inhibition activity)	99.591	93.281	97.570±0.12	105.901

[a]Values are expressed as mean ± standard deviation (n = 3)

Table 7. Chemical composition of optimized *Piper Betle* extract by Gas chromatography/Mass spectroscopy

Peak No.	Compounds	Chemical formula	Molecular weight	Retention time	Peak area %	Concentration (mg/mL)
1	Eugenol	$C_{10}H_{12}O_6$	164	10.51	11.92	0.012
2	Hydroxychavicol	$C_9H_{10}O_2$	150.17	11.19	66.55	0.067
3	Phenol, 2-methoxy-4-propenyl-, acetate	$C_{12}H_{14}O_3$	206.24	11.56	2.90	0.003
4	4-allyl-1,2-diacetoxybenzene	$C_{13}H_{14}O_4$	234.25	12.28	3.21	0.003



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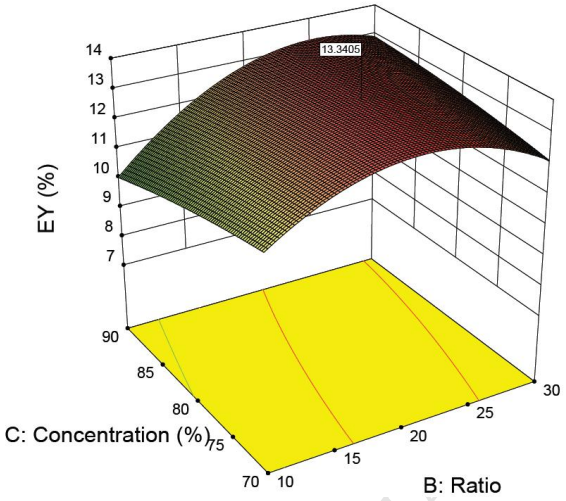

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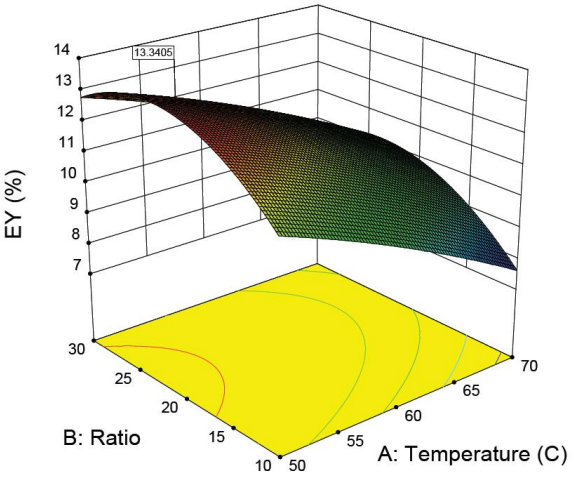

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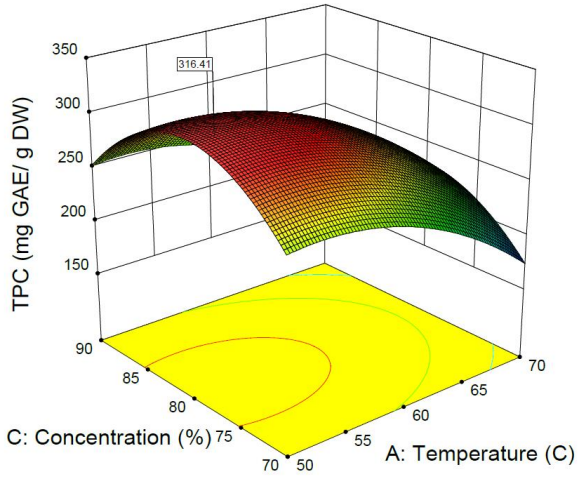

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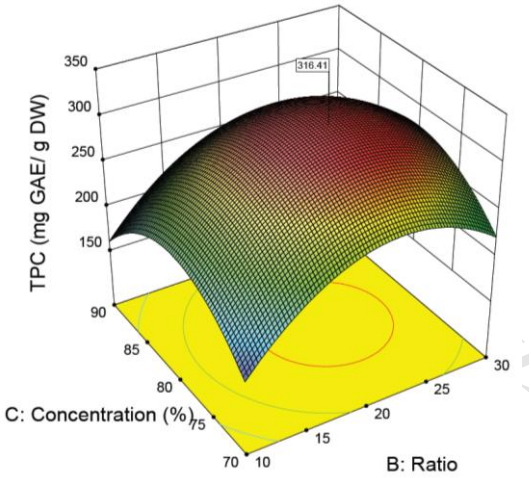

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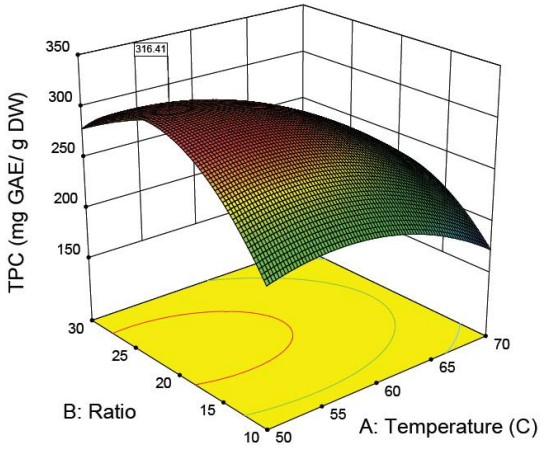

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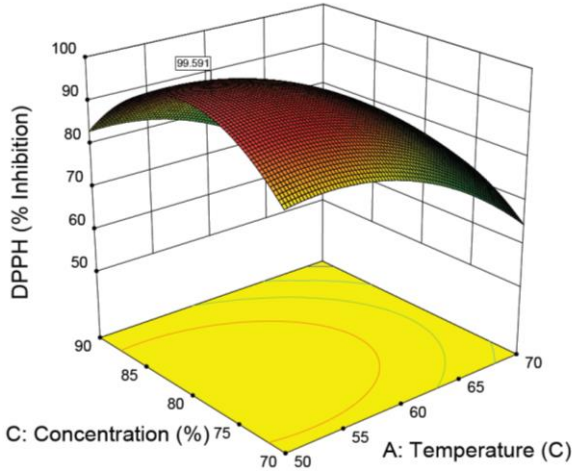

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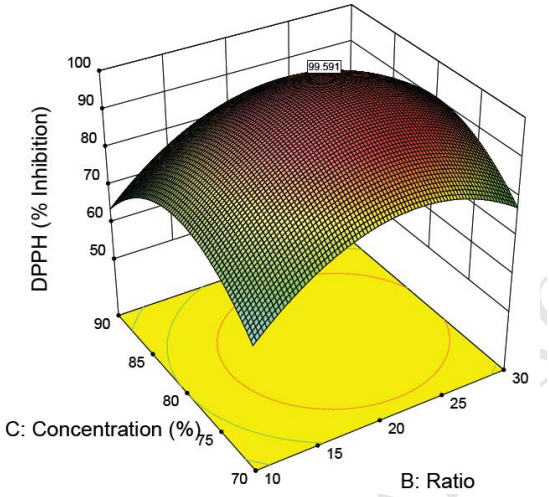

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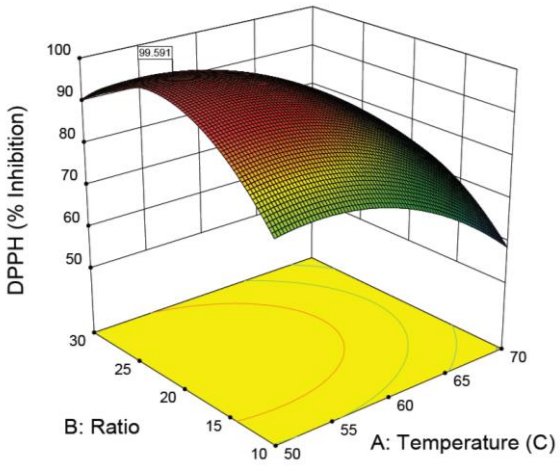

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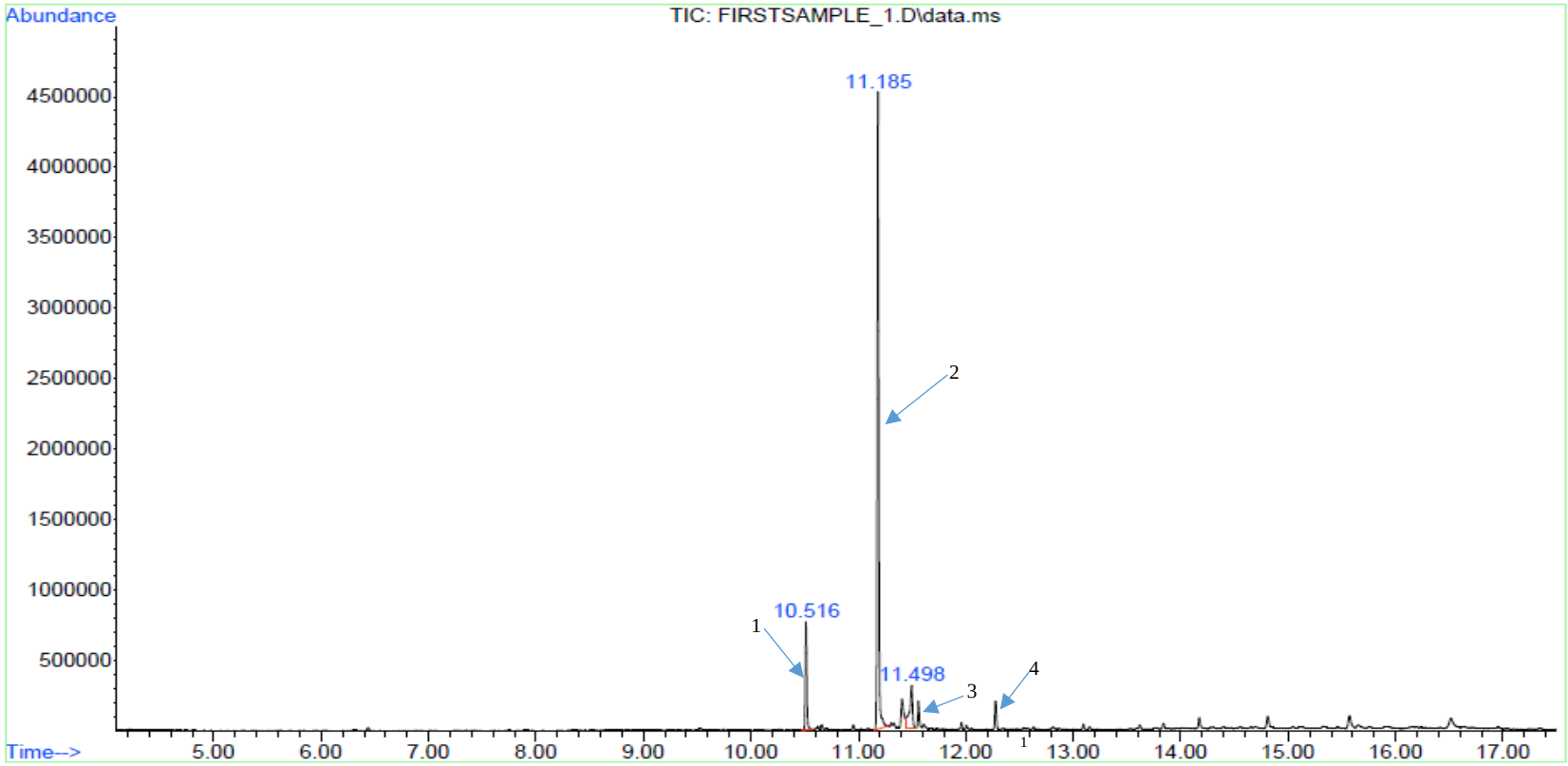
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Highlights

- Optimization of Ultrasound-assisted extraction of antioxidants from *Piper betle*
- Optimized condition at 51.60 ^{o}C with 78.74% ethanol concentration and ratio of 1:21.85 g/mL
- Phytochemical screening revealed additional constituents in ultrasound extracts
- Hydroxychavicol, eugenol, isoeugenol and 4-allyl-1,2-diacetoxybenzene were identified *via* GC/MS